Methods: We prepared primary human subchondral osteoblasts using the sclerotic medial portion of the tibial plateaus of OA patients undergoing total knee arthroplasty, or from tibial plateaus of normal individuals at autopsy. The expression of Rspo-1 and -2 was evaluated by qRT-PCR and their protein production by Western blot analysis. Wnt/β-catenin signaling was evaluated using two approaches: 1) target gene expression was measured using the TOPflash TCF/lef luciferase reporter assay, and 2) intracellular signaling partners β-catenin, phospho β-catenin, GSK3β and phospho GSK3β levels were evaluated by Western blot analysis. Mineralization in response to Wnt3a in presence or not of Rspo-2 was evaluated by Alizarin red staining.
Results: Rspo-1 expression was similar in normal and OA Ob. In contrast, the expression of Rspo-2 was reduced in OA samples compared to normal. Similarly, Western blot analysis revealed that Rspo-2 was reduced in OA Ob compared to normal. TGF-β1 reduced Rspo-2 expression and production whereas BMP-2 increased them. Wnt3a-dependent TOPflash TCF/lef reporter assay was reduced in OA Ob compared to normal. Recombinant human Rspo-2 alone failed to trigger Wnt signaling in OA Ob measured using the TOPflash reporter assay however, it stimulated about two-fold the Wnt3a-dependent response in these cells. Likewise, β-catenin levels which increased in presence of Wnt3a alone were further increased by Rspo-2 treatment. Rspo2 treatments also modulated the Wnt3a-dependent phospho GSK3β response of OA Ob. Wnt3a alone increased the basal mineralization of OA Ob and although Rspo-2 alone failed to modify the abnormal mineralization of OA Ob, it further increased the Wnt3a-dependent mineralization of OA Ob.
Conclusions: These data demonstrate for the first time that Rspo-2 is a new key player in abnormal OA Ob function. Reduced Rspo-2 levels in OA Ob are responsible, at least in part, for their reduced Wnt/β-catenin signaling and abnormal mineralization. As Rspo-2 is a secreted soluble protein, this could lead to potential new avenues of treatment of OA to correct their abnormal bone phenotype and mineralization.

059

GREM1, FRZB AND DKK1 ARE HIGHLY SPECIFIC MARKERS FOR ARTICULAR CARTILAGE WHICH PREVENT HYPERTROPHIC DIFFERENTIATION

J. Leijten[1], J. Emons[2], C. Sticht[3], E. Dekker[4], G. Rappold[4], C. van Blitterswijk[1], J.M. Wit[2], M. Karperien[1]
[1]Univ. Twente, Enschede, Netherlands; [2]LUMC, Leiden, Netherlands; [3]Univ. of Heidelberg, Heidelberg, Germany; [4]Univ. of Heidelberg, Mannheim, Germany

Purpose: Chondrogenically differentiated human mesenchymal stromal cells (hMSCs) are prone to undergo endochondral ossification. Under normal circumstances, this process is limited to growth plate cartilage (GP) but not articular cartilage (AC). The molecular mechanisms that prevent AC from terminal differentiation and ossification are largely unknown.
Our key objectives were i) to identify genetic markers that distinguish AC from GP, and ii) to identify molecular mechanisms that prevent AC from endochondral ossification. We hypothesized that this information can be utilized to optimize tissue engineering strategies to obtain AC from differentiating hMSCs.
Methods: Gene expression profiles of healthy AC and GP from the same patients were compared in a genome wide micro-array study. Protein expression was investigated for three articular enriched genes using Immunohistochemistry on AC and GP. Recombinant proteins of the same three articular enriched genes were added to chondrogenically differentiating hMSCs to investigate their effect on hypertrophic differentiation.
Results: We identified differential expression of 2915 genes between GP and AC, from which we generated a marker-set able to distinguish the two hyaline cartilage subtypes. Furthermore, we showed that differentiating MSCs acquire a genetic fingerprint resembling GP, but not AC.
Interestingly, the three most differentially expressed genes, being enriched in the AC, are Wnt- and BMP antagonists: FRZb, DKK1 and Gremlin1. Immunohistochemistry demonstrated the presence of these antagonists throughout the entire AC. In the GP their expression was mainly restricted to the resting zone.
Chondrogenically differentiated hMSCs did not express the 3 antagonists and underwent hypertrophic differentiation. Remarkably, the addition of recombinant Grem1, FRZb or DKK1 did not affect chondrocyte differentiation but prevented their hypertrophic differentiation.
Conclusions: Micro-array analysis has identified genetic fingerprints highly specific for the two types of hyaline cartilage, GP and AC, which can be used to optimize tissue engineering strategies. We have identified the Wnt- and BMP-antagonists, FRZb, DKK1 and Grem1 as specific markers for AC. We show that these antagonists prevent hypertrophic chondrocyte differentiation.
Our data suggest that Wnt- and BMP-antagonists play a prominent role in establishing a joint micro-environment, which prevents AC from undergoing endochondral ossification.

060

MOLECULAR NETWORK ON THE C/EBP-BETA AXIS INCLUDING RUNX2, MMP13, AND HIF2A CONTROLS OSTEOARTHRITIS DEVELOPMENT

M. Hirata[1,2], F. Kugimiya[1], A. Fukai[1], T. Saito[1], F. Yano[2], T. Ikeda[1], K. Nakamura[1], U.-i. Chung[2], H. Kawaguchi[1]
[1]The Univ. of Tokyo, Sensory & Motor System Med., Tokyo, Japan; [2]The Univ. of Tokyo, Ctr. for Disease Biology and Integrative Med., Tokyo, Japan

Purpose: CCAAT/enhancer-binding protein β (C/EBPβ) is a crucial transcription factor for chondrocyte differentiation and cartilage degradation in the process of endochondral ossification during skeletal growth. Since the endochondral ossification process is also known to be important for osteoarthritis (OA) development, this study examined the role of C/EBPβ in OA development and explored the related molecular network.
Methods: We performed histological analyses of homozygous (-/-) or heterozygous (+/-) deficient mice in comparison with the wild-type littermates. An experimental OA model was created surgically by inducing instability in the knee joints of 8-week-old mice, and OA severity was quantified by the OARSI histopathology grade. Human joint cartilage was obtained as surgical specimens of total knee arthroplasty. For functional analyses, we created stable lines of human chondrogenic SW1353 cells with retroviral overexpression of the genes, and evaluated the endogenous expression of target genes by real-time RT-PCR. Screenings of upstream and downstream signals were performed using SW1353 and human non-chondorogenic HeLa cells transfected with luciferase-reporter constructs containing the promoters. For comprehensive promoter analyses, we performed luciferase assay, electrophoretic mobility shift assay (EMSA), and chromatin immunoprecipitation assay involving sequential immunoprecipitation (ChIP-reIP) using SW1353 and HeLa cells.
Results: The C/EBPβ expression increased with OA development in the knee joint cartilages of the mouse experimental model and human surgical specimens. This expression was co-localized with Runx2, another key transcription factor for endochondral ossification. Although *C/EBPβ*-/- mice exhibited severe dwarfism and *Runx2*-/- mice were embryonically lethal, both *C/EBPβ*+/- mice and *Runx2*+/- mice grew normally under physiological conditions. Under the OA induction, however, both +/- mice showed resistance to joint cartilage degradation with decreased expressions of endochondral ossification markers like COL10A1, ADAMTS4, 5, and MMP3, 9, 13, as compared to respective wild-type littermates. To know the molecular interaction, we generated the compound heterozygous deficient (*C/EBPβ*+/-;*Runx2*+/-) mice. Although the *C/EBPβ*+/-;*Runx2*+/- mice grew nornally, the resistance to OA development and decreases of the endochondral ossification markers were more enhanced than *C/EBPβ*+/- or *Runx2*+/- littermates. Among the endochondral ossification markers, co-transfection of C/EBPβ and Runx2 most strongly enhanced promoter activity and endogenous expression of MMP13. Analyses of the *MMP13* promoter by luciferase assays identified the core responsive element as being located between the -144 and -89 bp region containing a C/EBP-binding motif (-103/-99) and a Runx-binding motif (-138/-132). Specific and direct bindings of C/EBPβ and Runx2 proteins to respective motifs were confirmed by EMSA and ChIP-reIP assay. Interestingly, the binding of C/EBPβ or Runx2 was blocked only when mutations were created in both motifs, but not by mutagenesis in either motif alone, indicating that these factors bind to respective motifs as a protein complex. We then explored the upstream signal using the*C/EBPβ* promoter assay among transcription factor families that are known to regulate chondrocyte differentiation, such as Sox, Runx, other C/EBP, MEF2, SP/KLF, ATF, NF-κB, and hypoxia-inducible factor (HIF); and identified HIF2A as the most potent transactivator of C/EBPβ. Our further promoter analyses in the *C/EBPβ* promoter identified a hypoxia-responsive element (HRE) motif (-69/-65) as the core responsive region.
Conclusions: C/EBPβ plays a central role in cartilage degradation during OA development by forming a molecular network with Runx2 as the co-factor, MMP13 as the target, and HIF2A as the inducer. This signal on the C/EBPβ axis may represent a therapeutic target for OA.